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Comparison of pro-inflammatory cytokines (IL-6, IL-1α and IL-1β) released by MPI and MARCO (-/-) knockout cells when stimulated by heat killed fungi- *Candida albicans and Aspergillus niger*

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Abstract

Background: Alveolar Macrophages serve the first line of defence against invading pathogens within the lungs. During the innate immune response, they recognise pathogens such as *Candida albicans* and *Aspergillus niger* as "non-self", phagocytosing them before the pathogen can infiltrate the body and cause disease. There are many Pattern Recognition Receptors (PRRs) that detect specific patterns on the pathogen (PAMPs), inducing an immune response which recruits different immune cells to the site of infection, releasing many cytokines that help with recruiting and clearance of the pathogen. MARCO is a Scavenger receptor that has an important role in the recognition and response mechanisms and this experiment was carried out to confirm the role of MARCO receptors on MPI cells stimulated with heat killed fungi.
Method: MPI cells were cultured, exposed to different concentrations of *C.albicans, A.niger* and Beta-glucan over a period of 16-18 hours and then quantified using ELISA. The cytokines measured were IL-6, IL-1α and IL-1β. The same procedures were carried out on MARCO-/- MPI cells too.
Results: The results show a significant difference in the amount of pro-inflammatory cytokines produced compared to the control, demonstrating that the immune response was instigated.
MARCO-/- cells showed no immune response compared to the control. They were also compared to the normally stimulated MPI cells.
Conclusion: The study consolidates the importance of the MARCO receptor in response to pathogens.

Keywords: Alveolar Macrophage, MPI cells, β-glucan, LPS, Pro-inflammatory Cytokines, *Candida albicans, Aspergillus niger,* PRRs, PAMPs, MARCO, Scavenger Receptors

Introduction

Macrophages

Heterogeneous population of myeloid cells (3) that act as the primary defence against invading microorganisms are known as macrophages – the "big eaters" of the immune system within the body (4, 5) (6). In the presence of Macrophage–Colony stimulating Factor (M-CSF) (and GM-CSF), they develop from Haematopoietic stem cells (HSCs) within the Bone Marrow (BM), differentiating into subsets for specific tissues around the body, although some stay for general purposes, predominantly used in the innate immune response (5). GM-SCF is an important haematopoietic growth factor, as well as an immune modulator, produced by a various types of cells including T-cells, macrophages, endothelial cells and fibroblasts upon receiving an immune response. GM-CSF can work in a paracrine way, recruiting leukocytes and other immune cells to enhance the host defence mechanisms. The growth of progenitor cells and M-CSF are thought to enhance the number of cells as well as regulate the differentiation and functional roles of the macrophages (4).

Pathogen Associated Molecular Patterns (PAMPs) and Pattern recognition receptors (PRRs)

The constant threat of pathogen invasion has caused the both, Innate and adaptive, parts of the immune system to evolve. This evolvement is to eliminate infective microorganisms (7). Pathogen Associated Molecular Patterns (PAMPs) are recognised by Pattern Recognition Receptors (PRRs) like Toll-like receptors (TLRs) on immune cells (5). The PPRs are essential, as they help initiate an immune response against the pathogen. They also give some form of specificity to a generalised immune response, e.g., LPS, present on gram-negative bacteria, is recognised by TLR4 receptors by allowing the pathogens to not only be recognised as a bacteria but also the type (gram positive/gram negative etc.), as shown in table 1(6).

Table 1: Different membrane bound receptors and the microbial components that correspond to specific Bacterial, Viral or fungal species (6, 7)

Receptor	Microbial components and Species	Location
Toll-Like receptors		
TLR2	Peptidoglycan (PG)- Gram Positive bacteria Lipoteichoic acid (LTA) – Bacteria B *Streptococcus* Phospholipomannan – *Candida albicans* Mannan – *Candida albicans*	Cell surface
TLR4	LPS – Gram negative bacteria	Cell surface
C-Type lectin receptors		
Dectin-1	Zymosan –Fungus β-glucans and *Saccharomyces cerevisiae*	Cell surface
Scavenger receptors:		
MARCO	LPS- Gram Negative bacteria LTA- Group B *Streptococcus*	Cell surface

Active macrophages have different physiological roles, performing distinct immunological functions (8). During events of stress or damage, physiological changes take place within the macrophage, which instigates pro- inflammatory mediators such as Tumour necrosis factor Alpha (TNFα), Interleukin- 6 (IL-6), Interleukin- 1α (IL-1α), Interleukin-1β (IL-1β) and anti-inflammatory mediators such as Interleukin-10 (IL-10) to be released (5, 6).

Alveolar macrophages play an important role in protecting the lungs from pathogen attack, leading to respiratory disorders caused by pathogens such as Aspergillus niger. There is a great need to develop anti-fungal drugs to combat against fungal infections, which are on the increase worldwide. The increases in drug-resistant strains of fungi lead to more worrying concerns for the future (1). Other sources of data mention the correlation of inhaled Particulate Matter (PM) causing inflammation within the airways and lungs. They have also linked this systemic inflammation with impacts on the coronary arteries, giving rise to adverse cardiovascular problems (2).

Toll-like Receptors (TLRs)

The TLR family have receptors that reside on the cell surface and within the cell, emphasising the specific tasks they undertake. As determined in some studies, the specificity of TLRs accommodates various isoforms of Lipopolysaccarides (LPS) to discriminate between self and non-self. Sometimes, ligands that are present intracellularly are recognised by TLRs or by TLR co-receptors, e.g. CD36, which may consequently act as danger signals at the site of damage (9)

C-type lectins (CTL)

C-type lectins (CTL) are PRRs that recognise carbohydrate structures of fungi as PAMPs. They also recognise PAMPs involved against infection in the host (10). The fungal walls are mainly composed of multiple layers of carbohydrates, which include mannans, β-Glucan and chitins, which are all recognised by the C-type lectin receptors (10) (6).

Pro-inflammatory cytokines

In this study, IL-6, IL-1α and IL-1β are the three cytokines that will be measured. The cascade initially is prompted by the PAMPs – PRR complex (pathogen-receptor complex), inducing an innate immune response. As a result of this response, different cells are recruited to the site of infection and different cells produce various cytokines within the body (11). Often a cytokine shows overlapping functions, for example, IL-(6) is produced in events of infection or trauma, in which the elevated levels of IL-6 is usually associated with disease states within the body (12). It is a pro-inflammatory cytokine, which is not only involved in the response to infection, but also in regenerative, neural and metabolic functions within the body (11); it is also an anti-inflammatory cytokine, serving an important role during altered health states (12). Signalling intracellular is enabled by the homodimerization of glycoprotein 130 (gp130) as a consequence of a ligand-receptor complex being formed. Cell signalling within is induced via the activation of gp130 tyrosine kinases (JAK1, JAK2 andTYK2) along with the phosphorylation of STAT1 and STAT3 (12).

IL-1 secretion is found in patients infected by bacteria, virus, fungi or parasites (13). IL-1α and IL-1β trigger an inflammatory response initiated through Myd88 activation, inducing NF-κB, although recent findings show that they perform different activities within the body. IL-1β is another mediator of inflammation and occurs as a result of the host's response to infections. A raised amount of IL-1β is often linked with various diseases, for example, Type 2 Diabetes (T2D), atherosclerosis, Crohn's disease etc. It is also thought to be involved in neurodegenerative disorders such as Alzheimer's and Parkinson's disease (PD), in which the impairment has risen from inflammation following injury (14). While IL-1β has a beneficial role of mediating an immune response against the pathogenic infiltration, it can also assist and promote the pathogenesis of tissue damage that leads to chronic inflammatory disease (14). IL-1β is not initially expressed on the myeloid cells

derived from the BM (15). Initially, the IL-1β is synthesized in an inactive form (IL-1β pro), which is then processed to become biologically active via capsase-1, as a result of which it is then released into the extracellular environment. This processing and presentation of the active form of IL-1β has many underlying mechanisms, differing to the conventional secretory route via Endoplasmic reticulum- Golgi (ER-Golgi), and following a much more complex route (14) (16).

Unlike IL-1β, IL-1α can be found within the cell under normal conditions. It is in its active form as a precursor and a processed form. Its functionality is within the intracellular compartment, particularly the nucleus, but it affects the inflammation and immunity outside the cell. Therefore, like Il-6 and Il-1β, IL-1α can also be classed as a dual – function cytokine (15).

Scavenger receptors

Another class of PRRs is scavenger receptors (SRAs). This is a large class of proteins, which has the capacity for broad-spectrum ligand-binding consisting of 8 subclasses, that can bind to components of both Gram-positive and Gram-negative bacteria. Soon after the first SRAs were identified, it was evident that macrophages carry several variants of the receptor, with comparable and distinctive traits. Alveolar Macrophages (AMs) are the immune cells of the lungs that have a specific capacity for broad specificity ligand binding, responsible in eliminating pathogenic macromolecules that can be inhaled (17). Macrophage Receptor with Collagenous structure (MARCO) is a type of SRA that is found on AMs. Studies done on MARCO knockout mice demonstrated the importance of the role of SRAs, as it showed a higher susceptibility to bacterial pneumonia and vigorous inflammatory responses to inhaled particles from the environment (17).

Inflammasome activation

Intracellular multi-proteins that play a pivotal role in activating the immune system are the inflammasomes. The detection of PAMPs and danger-associated molecular patterns (DAMPs) are the primary functions of the inflammasome. Inflammasomes are molecular platforms responsible for the activation of inflammatory capsases, 1 and 5 which leads to the secretion of pro-inflammatory cytokines IL-1β and IL-18, stimulating a potent inflammatory response (18). Muramyl dipeptide, a breakdown product of peptidoglycan from the bacterial cell wall is the only component that is known to activate the NACHT, LRR and PYD domains-containing protein 3 (NALP3) inflammasome (18)

Candida albicans and Aspergillus niger

Currently, one of the major health issues is infections caused by fungi, both superficial and invasive. The incidences of these infections are rising worldwide due to increasing number of immunocompromised individuals as well as Antibiotic resistant strains, with *Candida albicans (C.albicans)* and *Aspergillus fumigatus (A.fumigatus)* being the leading causes of fungal disease (19). The expression and secretion of IL-1β, with the help of IL-17 producing helper T cells is significant in the defence mechanism against the fungal infections, although very little is known about the control mechanisms involving IL-1β after the infection has passed (19). *C. albicans*, via Dcetin-1 induces T-helper 1 and 17 cells which, in turn, activate transcription factor NF-κB. This is a pathway dependent on kinase Syk, instigating the activation and assembly of distinct inflammasomes. A variety of infectious signals (PAMPs) trigger different responses that are recognised by particular PRRs but it is still unclear if and how the cytosolic sensors sense fungal infections (19).

Aspergillus niger (A. niger) is a filamentous fungi, that inhabits the soil (20, 21), known for its importance in biotechnology, especially in food manufacturing However, it may also produce spores that are harmful to humans (20)). It is also used in many functional studies to investigate the components that contribute to the majorly of fungal secretion pathways (21), adapting to its extreme environmental conditions (22).

The aim of this study is to observe the cytokine release in response to stimulated MPI cells with Heat-killed fungi and compare it to the MARCO SR-/- MPI cells, determining the importance of the SRs in the initiation of an immune response.

Materials and methods

Max Planck Institute (MPI) cells

These were cultured from unseparated fetal liver cells in the presence of GM-CSF, following the protocol from Fejer *et al,* 2013. They were cultured in RPMI (10% FBS, 1% Penicillin/ streptomycin and 1% L-glutamine), at 37°C and 5% CO_2 humidified atmosphere. Cells that were already differentiated into macrophages were provided by Dr. Fejer,

MARCO scavenger receptor knockout cells

Transgenically modified mice were used to produce MARCO deficient MPI cells (MARCO knockout cells), cultured and supplied by Dr. Fejer, Plymouth University (unpublished results (5)). Cultured in RPMI (10% FBS, 1% Penicillin/ streptomycin and 1% L-glutamine), at 37°C and 5% CO_2 humidified atmosphere.

Heat killed fungi

The heat-killed fungi for this experiment were provided by Matthew Emery, Senior Microbiology technician, Plymouth University.

Candida albicans is from the National Collection of Pathogenic Fungi (NCPF), strain number NCPF 3179. It was grown in Saboraud dextrose broth, washed 3 times through centrifugation and suspension in PBS. It was then heat killed at 60°C for 45 minutes.
Aspergillus niger is an environmental isolate from the university culture collection (no strain number). Grown in Saboraud dextrose agar for 9 days, the spores were harvested in PBS-tween solution. It was washed the same way as *C. albicans*. It was then heat-killed at 68°C for 4 hours because the sores are significantly more difficult to kill.
The fungi were stored at 4-6 C (normal refrigerator temperature).

Reagents

Cell culturing (protocol 2, appendix):
The MPI cells were cultured on a regular basis (2-3 times a week). All plastic ware for cell culture and Virkon for discarding the waste material were purchased from VWR. The cells were cultured using PBS, EDTA and RMPI medium supplied by Lonza. MPI cells were cultured in RPMI medium (10% FBS, 1% Penicillin/ streptomycin and 1% L-glutamine), at 37°C and 5% CO_2 humidified atmosphere (5).

GM-CSF was extracted from the X63 GM-CSF producing cell line (recombinant GM-CSF may also be used). Supernatant was extracted after 7 days of cell culture, filter sterilised and then a titration method was used to see what concentration to use for optimal cell

growth. 120µl was used for 12ml of the cultured cells (75m^3 flask). GM-CSF was stored at -20°C, and a freeze/thaw cycle was avoided.

1,6-D β-glucan (β-glucan) (Lieber), was supplied by Prof. Simon Davies, Fish Nutrition, Plymouth University.

LPS was extracted from an E-coli strain that was manufactured in the Max Planck Institute (Smooth form). Prepared and purified following the protocol from Fejer *et al* (5*)*.

Cytokine ELISAs
The primary and secondary antibodies for IL-6, IL-1α, IL-1β (all 0.5µg/ml stocks) were purchased from Ebiosciences, along with Avidin (diluted 1:500) and Super Aqua blue. The BSA (2%) was from sigma for the blocking of wells. All ELISA's were performed according to manufactures' protocols (protocol 3, appendix). The ELISA plates were read using Molecular devices - Versa Max reader, using the software, Softmax Pro.

Statistical analysis
Statistical analysis of the data was performed on Minitab 16 software. One-way ANOVA with Levens test was used to test the homogeneity of the variance followed by Tukeys test with 95% significance level. P-values of ≤0.05 were considered significant.

Results

IL-6 secretion by MPI cells stimulated by Heat killed fungi and β-glucan

Figure 1 shows the secretion of IL-6 by MPI cells stimulated with different concentrations of Fungi and β-glucan. Different samples of stimulated MPI cells were used, stimulated at different times, on different days and both samples were taken into considerations (n=2). Statistical evidence, as shown in the figure 1 (graph A, B and C) show an overwhelming difference in the amount of IL-6 produced by both plates stimulated by heat-killed *C.albicans* stimulated MPI cells when compared to the control and there is a similar response when MPI cells were stimulated by *A. niger ,* suggesting that an immune response was induced.

Literature suggests that *Candida albicans* is picked up by TLR2 and dectin-1 receptors causing inflammatory and immune regulation responses (23), which is what is evident in fig. 1(A), where *C. albicans* induces a highly significant response.

Aspergillus candida, passively escaping the physical barrier of the respiratory tract can cause effector mechanisms to induce the innate immune response by AMs and Dendritic cells (DCs) and other immune cells, releasing cytokines and chemokines, e.g., IL-6 as demonstrated in graph B (fig 1) (24).

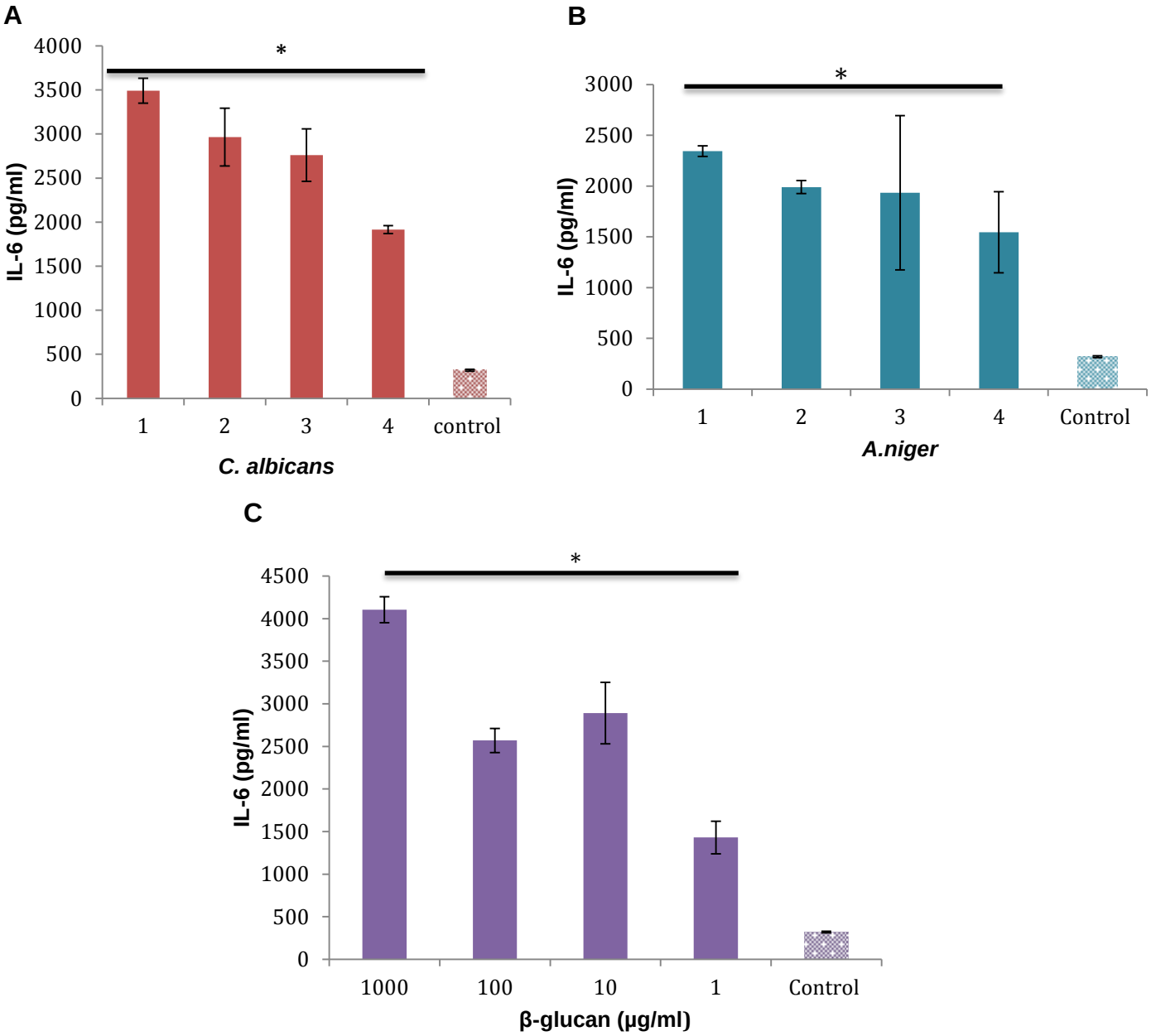


Figure 1. In vitro secretion of IL-6 from MPI cells when stimulated by Heat killed Fungi and Beta-glucan at different concentrations.
IL-6 secretion of MPI cells stimulated with Heat killed *C. albicans* and *A.niger* and also β-glucan (A, B and C respectively), with four concentrations and the control which had no cells- $5.5x10^4$ Infectious Particles (IP) (1), $5.5x10^3$ IP (2), $5.5 x10^2$ IP (3) and 5.5x10 IP (4). It was quantified by ELISA after 22 hour stimulation and the average IL-6 secretion were taken into consideration for the different concentrations (1-4). *C. albicans* IL-6 secretion compared to control – ($p<0.001$)($n=2$). *A. niger* IL-6 secretion compared to control ($p<0.001$)($n=2$). β-glucan IL-6 secretion compared to control ($P<0.05$). When comparing all the different stimuli to one another, the tests analysis showed no difference between them ($p>0.10$)($n=2$). The data shown are means ±SEM of 3 independent experiments, each consisting of 2 repeats. (one-way ANOVA followed by Tukeys analysis) at 95% confidence levels – asterisks indicate significant difference (*).

The highest concentration for β- glucan was 1mg/ml (1); it was diluted down ten-fold each time - 100µg/ml (2), 10µl/ml (3) and 1µg/ml (4). One way ANOVA shows significant difference in concentrations 1-4 compared to the control. The comparison to the control showed some significant difference. β- glucans, recognised by Dectin-1 (PRRs) expressed on DCs, macrophages and neutrophils, binds and upon binding, NF-κB which in turn leads to the production of cytokines as demonstrated in Graph C (Fig 1).

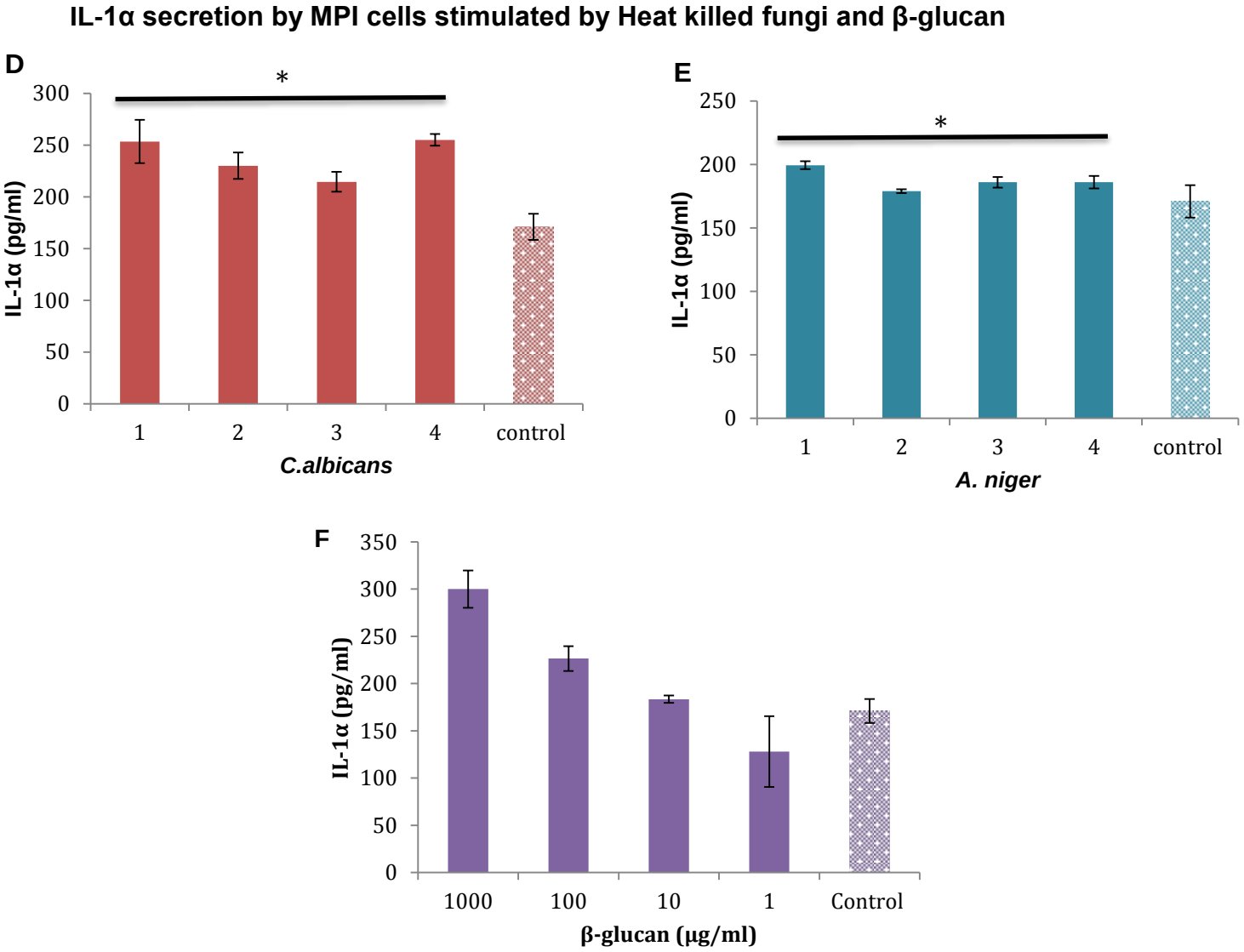


Figure 2. In vitro secretion of IL-1α from MPI cells when stimulated by Heat killed fungi and Beta-glucan at different concentrations.
IL-1α secretion of MPI cells stimulated with Heat killed *C. albicans* and *A.niger* and also β-glucan (D, E and F respectively), with four concentrations and the control which had no cells. *C.albicans* and *A. niger* had concentration 1-4 -5.5x10^4 Infectious Particles (IP) (1), 5.5x10^3 IP (2), 5.5 x10^2 IP (3) and 5.5x10 IP (4). It was quantified by ELISA after 16 hours of stimulation and the average IL-1α secretion were taken into consideration for the different concentrations. *C. albicans* IL-1α secretion compared to control ($p<0.001$)(n=2). *A. niger* IL-α secretion compared to control ($p< 0.01$)(n=2). β-glucan (β- glucan was 1mg/ml (1), it was diluted down ten-fold each time - 100µg/ml (2), 10µl/ml (3) and 1µg/ml (4)) IL-1α secretion compared to control ($p>0.10$)(n=2), The data shown are means ±SEM of 3 independent

experiments, each consisting of 2 repeats (one-way ANOVA followed by Tukeys analysis) at 95% confidence levels – asterisks indicate significance.

Figure 2 shows the IL-1α secretion when the MPI cells were stimulated overnight by different concentrations of Heat killed *C. albicans, A. niger* and Beta-glucan (graph D, E and F respectively). *C.albicans* stimulated MPI cells IL-1α secretion, when statistically analysed, shows overwhelming evidence of difference in the amount of cytokine produced when compared to the control (D). Furthermore, *A. niger* IL-1α secretion showed some evidence of difference compared to the control (E). The IL-1α produced was stored within the cell and was released following cell lysis (25). Concentration 1-4 of β-glucan show no evidence of difference compared to the control of β-glucan stimulated MPI cells (F). Via the Dectin- 1 receptors on AMs, the binding of Beta-glucan to the macrophages would lead to phagocytosis, releasing the IL-1α (26).

IL-1β secretion by MPI cells stimulated by Heat killed fungi and β-glucan

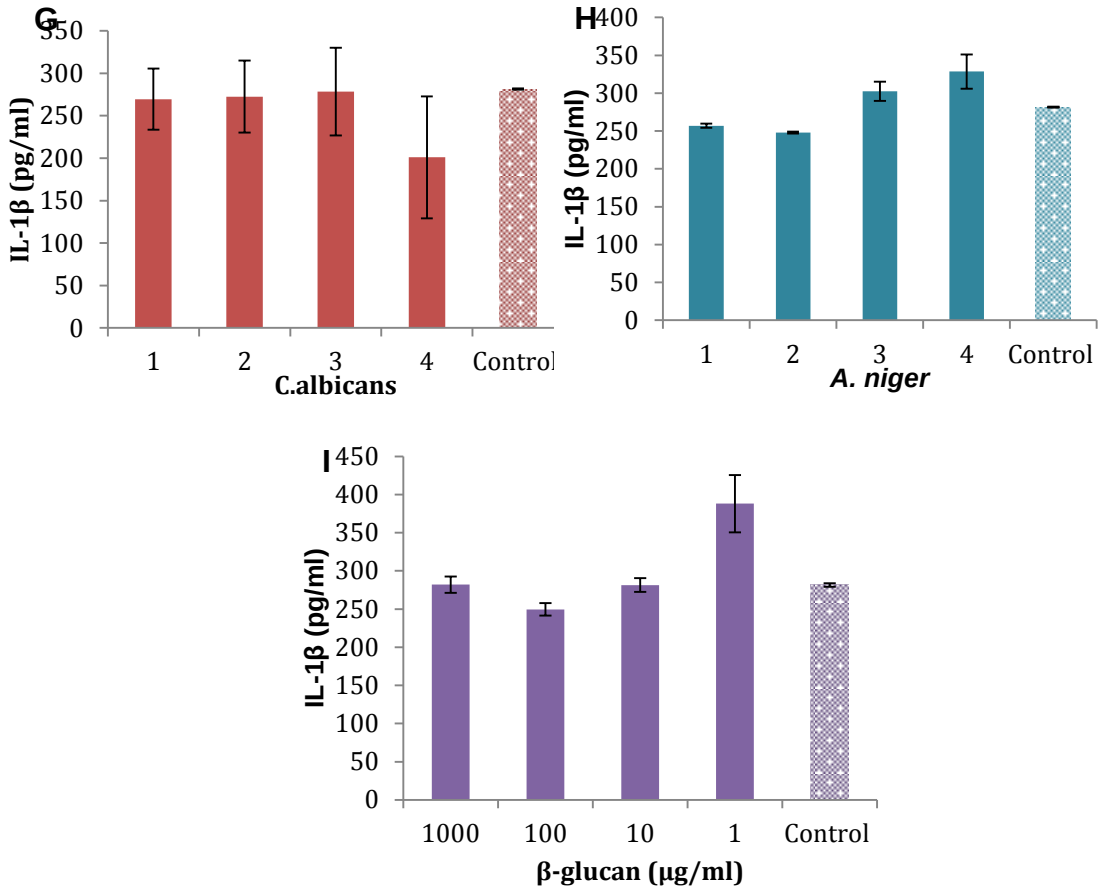


Figure 3. In vitro secretion of IL-1β from MPI cells when stimulated by Heat killed fungi and Beta-glucan at different concentrations.
IL-1β secretion of MPI cells stimulated with Heat killed *C. albicans* and *A.niger* and also β-glucan (G, H and I respectively), with four concentrations and the control which had no cells. *C.albicans* and *A. niger* had concentration 1-4 -5.5x10^4 Infectious Particles (IP) (1), 5.5x10^3 IP (2), 5.5 x10^2 IP (3) and 5.5x10 IP (4). Stimulated for 16 hours and quantified by ELISA after 24 hours of stimulation and the average IL-1β secretion were taken into consideration for the different concentrations. *C. albicans* IL-1β secretion compared to control (p>0.10)(n=2). *A. niger* IL-1β secretion compared to control (p>0.10)(n=2). β-glucan IL-1β secretion compared to control (p>0.10)(n=2), The data shown are means ±SEM of 3

independent experiments, each consisting of 2 repeats. (one-way ANOVA followed by Tukeys analysis) at 95% confidence levels.

Figure 3, graphs G, H and I show no visual pattern and when statistical analysed (One way ANOVA) it showed that *C. albicans* (G), *A. niger* (H) and β-glucan (I) all display no / little evidence of difference when comparing the different concentrations of the fungi and β-glucan to the control. The results are consistent with previous data (27), showing significant responses within the different Aspergillus spp. in the amount of cytokines produced. It was discovered that only *A. fumigatus* and *A. flavus* induce a significant amount of TNF alpha and IL-1. On the other hand, *A. niger* was less effective along with *A. terreus* which is clearly evident in Fig 3 (H).

IL-6, IL-1α and IL-1β secretion by MARCO-/- MPI cells stimulated by Heat killed fungi and β-glucan

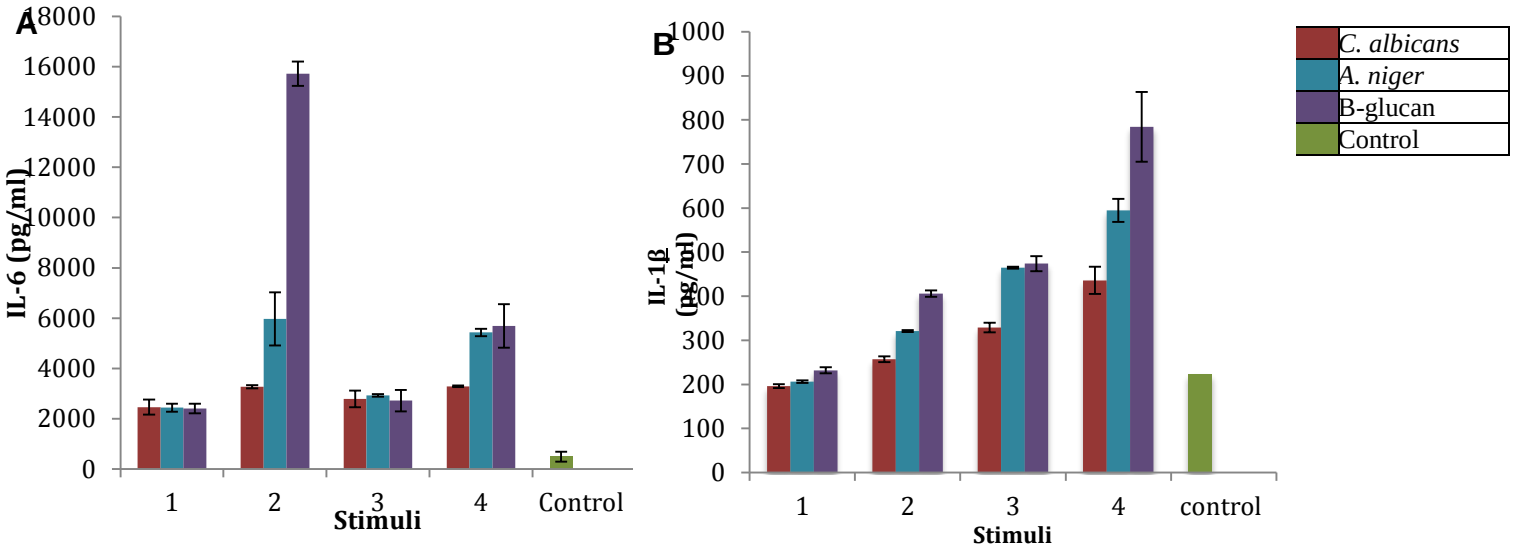


Figure 4. IL-6 and IL-1β release when MARCO ˉ/ˉ MPI cells are stimulated with Heat killed fungi and β-glucan at different concentrations (1-4).
IL-6 secretion by MARCO-/- MPI cells (A) stimulated by *C. albicans, A. niger and* Beta-glucan. Statistical analysis of the stimulated cells compared to the control (One way ANOVA, followed by Tukeys analysis) ($p>0.10$), n=1, - at 95% confidence level. IL-β secretion by MACRO-/- MPI cells (B) when stimulated by heat killed *C.albicans, A.niger* and Beta-glucan (J) (refer to key) compared to control and to the different stimuli. *C.albicans, A. niger* and β-glucan stimulated MARCO-/- MPI cells IL-1β secretion compared to the control ($p>0.10$), n=1, (One way ANOVA, Tukeys analysis) – at 95% confidence level. The data shown are means ±SEM of 3 independent experiments, each consisting of1 repeat1. *C.albicans* and *A. niger* -$5.5x10^4$ Infectious Particles (IP) (1), $5.5x10^3$ IP (2), $5.5 x10^2$ IP (3) and 5.5x10 IP (4). β- glucan- 1mg/ml (1), it was diluted down ten-fold each time - 100µg/ml (2), 10µl/ml (3) and 1µg/ml (4).

Figure 4 shows the average IL-6 secreted by MARCO-/- MPi cells when stimulated by the heat-killed fungi and β-glucan (A). The levels of IL-6 released show no evidence of difference between any of the stimuli or compared to the control.

IL-1β released by MARCO-/- MPI cells stimulated by *C. albicans, A.niger* and β-glucan (B). IL-1β levels released show no significant difference between the two species of fungi, *C. albicans* and *A. niger*,, and β-glucan compared to the control.

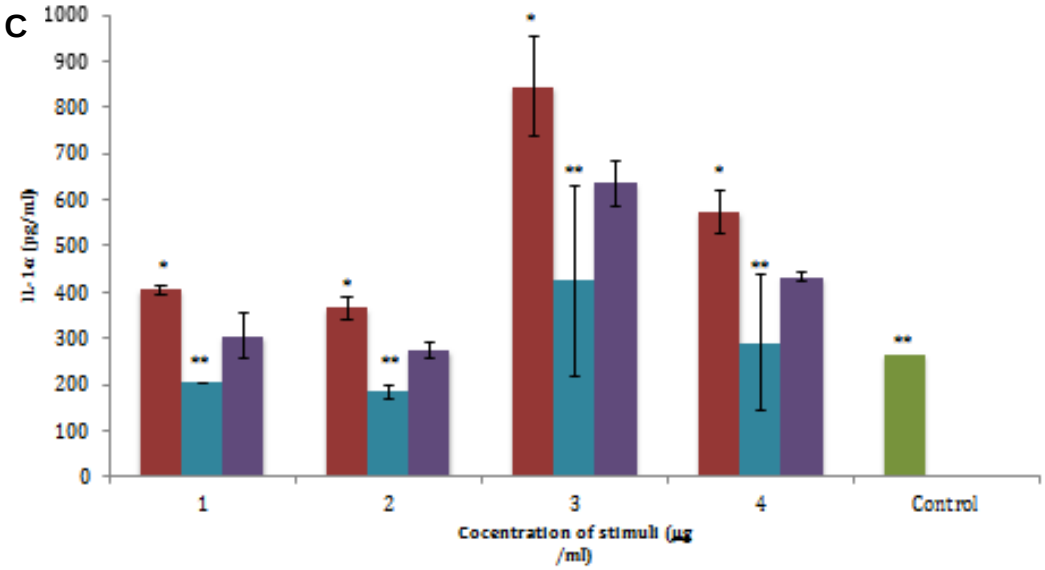
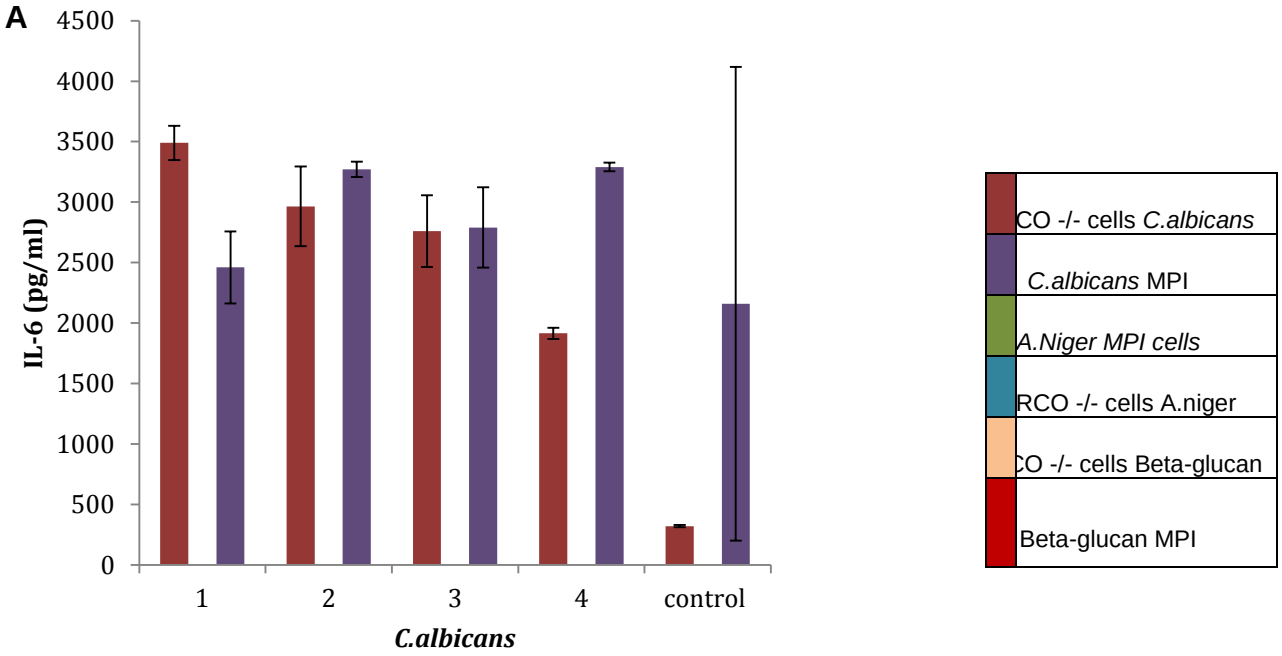


Figure 5 displays the average IL-1α released by MARCO-/- MPI cells stimulated by *C. albicans, A.niger* and Beta-glucan (C). There is some evidence to suggest difference in the IL-1α levels in the cells stimulated by heat killed *C.albicans, A. niger* and Beta-glucan stimulated cells compared to the control. Tukeys analysis confirmed a significant difference between *C. albicans* (*) compared to *A. niger* (**) and the control (**). Although none of these significances can be visualised from graph C, the statistical analysis shows clear differences between the control and MARCO -/- MPI cells in some instances.

Comparison of Cytokines secreted between MARCO -/- MPI cells and Normal MPI cells



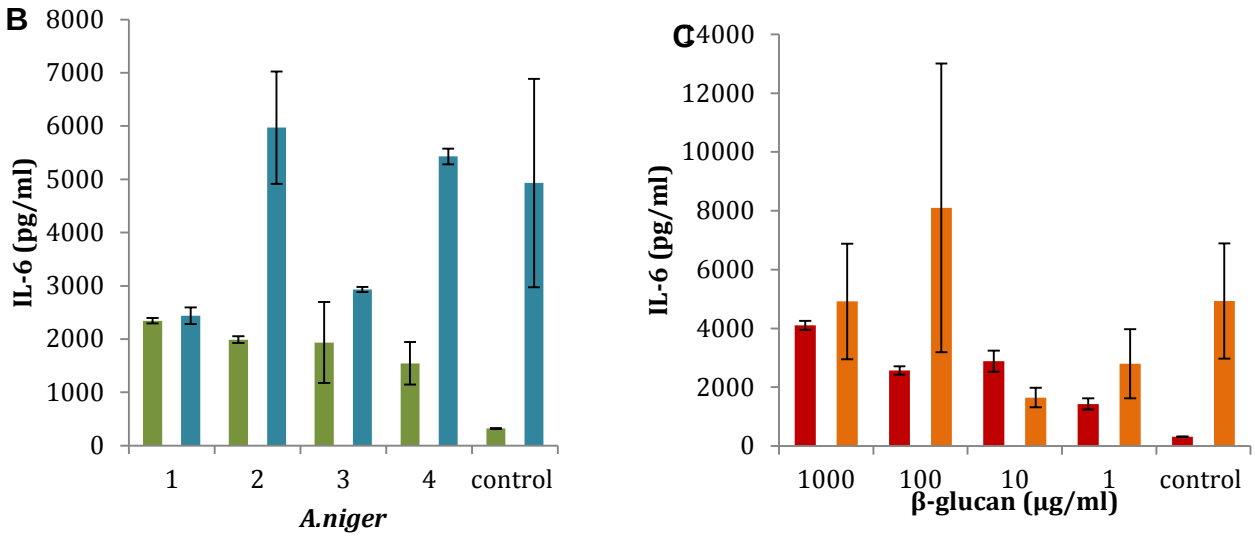


Figure 6: IL-6 secretion of MARCO-/- MPI cells compared to normal MPI cells stimulated cells.
IL-6 secretion by MACRO-/- MPI cells when stimulated by heat killed *C.albicans* (A)- ($p>0.10$), *A.niger* (B)- ($p>0.10$) and Beta-glucan (C)- ($p>0.10$) compared to MPI cells, at different concentrations (1-4) and the control ($p<0.05$)($n=2$). Analysed at 95% significance level (One way ANOVA, followed by Tukeys analysis). The data shown are means ±SEM of 3 independent experiments, each consisting of 1 repeat. *C.albicans* and *A. niger* -$5.5x10^4$ Infectious Particles (IP) (1), $5.5x10^3$ IP (2), $5.5\ x10^2$ IP (3) and 5.5x10 IP (4). β- glucan- 1mg/ml (1), it was diluted down ten-fold each time - 100µg/ml (2), 10µl/ml (3) and 1µg/ml (4).

Figure 6 shows the release of IL-6 secreted by MARCO-/- MPI cells that were stimulated with the same concentrations of stimuli mentioned above and compared to the MPI cells (normal). The IL-6 release was quantified by ELISA after approx. 18 hour stimulation, taking into consideration 2 repeats (n=2) for the normal MPI cells and one repeat for MARCO-/- MPI cells (n=1) due to the lack of time. Statistical analysis shows that there little/ no evidence of significance between MARCO-/- MPI cells stimulated with the different concentrations (1-4) and the control in comparison to the normal cells.

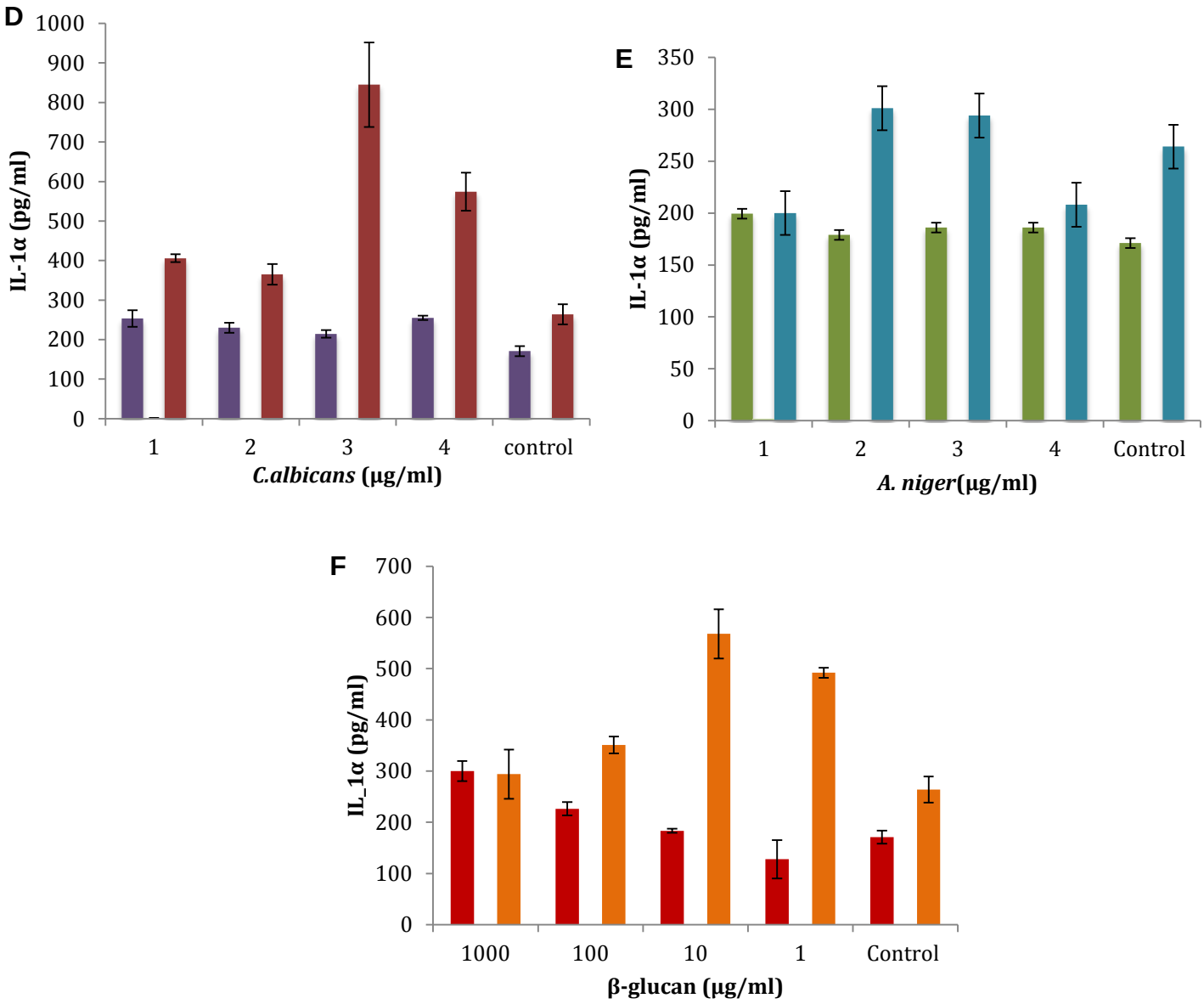


Figure 7. IL-1α secretion of *C.albicans* stimulated MPI cells and MARCO-/- MPI cells. IL-1α secretion by MACRO-/- MPI cells when stimulated by heat killed *C.albicans*(D)- ($p>0.10$) , *A.niger* (E)- ($p>0.10$) and Beta-glucan (F)- ($p>0.10$) compared to MPI cells at different concentrations ($p<0.05$) at 95% confidence level 9one way ANOVA, followed by Tukeys analysis) The data shown are means ±SEM of 3 independent experiments, each consisting of1 repeat1. *C.albicans* and *A. niger* -$5.5x10^4$ Infectious Particles (IP) (1), $5.5x10^3$ IP (2), $5.5 x10^2$ IP (3) and 5.5x10 IP (4). β- glucan- 1mg/ml (1), it was diluted down ten-fold each time - 100µg/ml (2), 10µl/ml (3) and 1µg/ml (4))

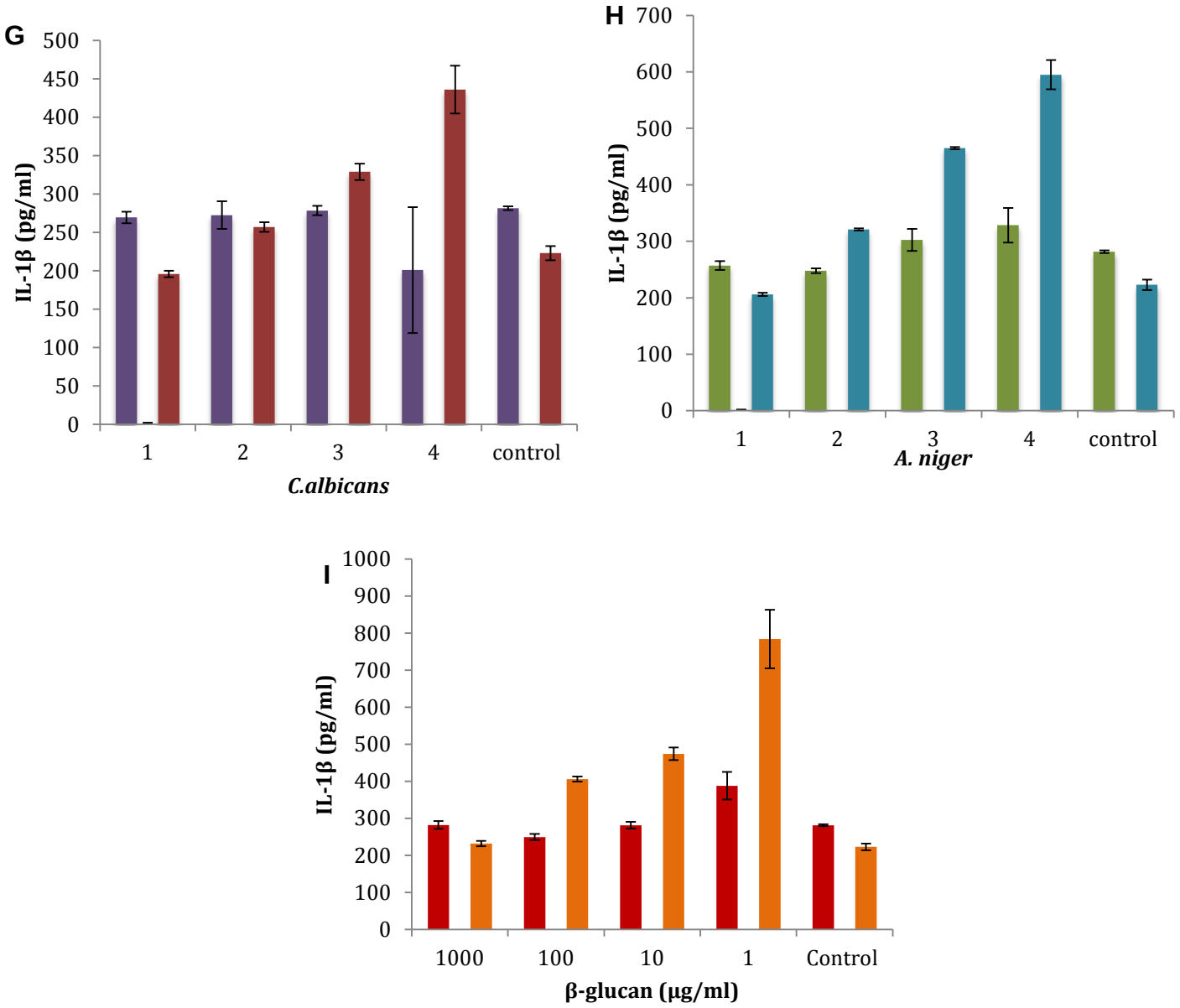


Figure 8: IL-1β secretion fungi stimulated MPI cells and MARCO-/- MPI cells.
IL-1β secretion by MACRO-/- MPI cells when stimulated by heat killed *C.albicans* (G)- (p>0.10), *A.niger* (H)- (p>010) and Beta-glucan (I)- (p>0.10) compared to MPI cells (One way ANOVA, followed by Tukeys analysis)- at 95% confidence level. The data shown are means ±SEM of 3 independent experiments, each consisting of 1 repeat
C.albicans and *A. niger* -5.5x10^4 Infectious Particles (IP) (1), 5.5x10^3 IP (2), 5.5 x10^2 IP (3) and 5.5x10 IP (4). β- glucan- 1mg/ml (1), it was diluted down ten-fold each time - 100µg/ml (2), 10µl/ml (3) and 1µg/ml (4).

The release of IL-1α (fig 7) and IL-1β (Fig 8) by MARCO-/- MPI compared to MPI (normal) cells that were stimulated with all three stimuli (concentrations 1-4) showed no significant difference in the amount of cytokines released, similar to fig 6.

In the recognition and clearance of fungal pathogens, a direct role for Scavenger receptors have not been established before but there is indirect evidence in studies carried out (1) showing evidence of the involvement of SRs recognising fungi.

Discussion

Interactions between pathogens and humans are more common than some may think, in fact, on a daily basis this interaction can be up to a million infectious agents, through different means of contact, ingestion and inhalation (29). As mentioned in the introduction, when a pathogen infiltrates the immunological barriers, the innate immune system, which is non-specific to a particular pathogen, creates a broad/ general response (29) when stimulated by particles that are recognised as 'non-self', to protect from infection.

The data collected shows that MPI cells, like AMs and Bone Marrow Derived Macrophages (BMDMs), instigates an immune response to the heat killed fungi as demonstrated in fig 1. Graphs A, B and C show significant difference in the amount of IL-6 produced at different concentrations (1-4) compared to the control, displaying the immune response that was triggered in the stimulated MPI cells (28). Although graphs B and C do not show an obvious visual pattern of decline in the cytokine produced, the data shows a slight decrease between the concentrations, which is not apparent when both samples (n=2) are averaged.

The IL-6 release (Fig1. Graph A and B) in both species of fungi showed a steady decrease in cytokine production as the concentration of the fungi decreased. This is what was expected as there are less Infectious Particles (IP) that can stimulate the MPI cells and, therefore, it can be concluded that concentration of the fungi is directly proportional to the amount of the IL-6 produced. The same trend is elicited in the MPI cells stimulated with β-glucan. Literature suggests that *Candida albicans,* an opportunistic fungus that resides in the human oral cavity and intestine, causes candidiasis when there is a commensal microbiome shift or the individual is immunocompromised. Before they escape from macrophages after phagocytosis, they are picked up by TLR2 and dectin-1 receptors causing inflammatory and immune regulation responses (21), which is what is evident in fig. 1, where *C. albicans* induces a highly significant response. β(1,3)-glucans and β(1,6)-glucans are important components of the *C.albicans* cell wall. It has been shown in previous studies (28) that heat treated *C. albicans* induces a significantly larger release of TNF-α, IL-6, IL-10 and IFN-γ (pro-and anti-inflammatory cytokines) than the live strain of the fungus. This is due to the disruption of the integrity of the cell wall, leading to the more exposed β-glucan that is recognised by Dectin-1 receptors (28). The use of heat-killed *C. albicans* enhanced the response of IL-6 secretion in Graph A (fig.1). The study went on to measure increase and decrease of the cytokines over time- TNFα concentrations peaked after 8-24 hours of stimulation followed by a sharp decline thereafter. On the other hand, IL-6 levels increased after the same time frame of stimulation, but the IL-6 secretions plateaued after 24-48 hours of stimulation (28). In relation to graph A (fig.1), the decrease in the Il-6 levels decreased slightly at each concentration (2-4) after 1 ($5.5x10^4$ IP).

In the *Aspergillus* spp., *Aspergillus fumigatus (A.fumigatus)* is the most common cause of disease, although, now, other species such as *A.niger* are also increasing to become more frequent causes of disease (24). Due to their small size, 2-5μm in diameter, the *Aspergillus* condida can passively escape the physical barrier of the respiratory tract. The particles avoid the cilia and travel into the body where it is recognised by various PRRs

and peptides that breakdown and clear the pathogen. The effector mechanisms induce the innate immune response by the alveolar macrophages (AMs) and other dendritic cells (DCs), recruiting more cells to the site of infection, increasing the release of cytokines such as IL-6 as demonstrated in graph B (fig 1) (24). The recognition of *A.niger* by the host is achieved via soluble recognition molecules along with receptors on immune cells. According to the study done on the innate response to *Aspergillus* spp., the condida maturation process leads to the removal of protein-hydrophobic layer, a significant morphological change, exposing the layer of the inner cell wall- composed mainly of β-glucans, mannan and chitin. The results of this data show evidence of insignificant inflammatory response induced by the resting condida- the immune response is only induced when the condida is live/ active (24). The response the immune system to resting condida, having no significant response might explain the trends seen on all the *A.niger* stimulated MPI cells (fig 1(B), Fig 2 (E) and Figure 3 (H)), as this experiment was done with heat-killed fungi. To improve understanding of the fundamentals of cytokines released by *Aspergillus* spp. stimulated cells, the experiment could be repeated with live strains of the fungi and also having more repeats in which to compare the response.

Innate immune response enables the activation of effector mechanisms on the AMs and DCs, leading to the recruitment of other immune cells. The condida in resting state become active (swollen) in 4-5 hours of being in the lungs. If it is not cleared, the swollen condida germinates to form hyphae in about 12-15 hours on the luminal side of the mucosal surface initiating a tangible inflammatory response (24).

The comparison of the IL-6 compared to the control showed some significant difference (Fig 1 (C), this confirms that an immune response was induced. β- glucans (PAMPs) being the major component of Fungal cell walls is recognised by Dectin-1 (PRRs) highly expressed on dendritic cells and in some macrophages and neutrophils. Upon interaction and binding of β-glucan, macrophages produce bactericidal compounds like lysozyme, reactive oxygen radicals and nitric oxide. In addition, these cells start producing a number of inflammatory cytokines that will interact with the surrounding macrophages and lymphocytes to initiate local and acquired specific immunity. Some of these cytokines are IL-1, IL-6, and TNFα. The 1.3-1.6-β-D glucan (Beta-S) that was provided had a polysaccharide content of 1.3-1.6-β-D glucan (>70%) along with Mannan (<2%) and chitin (<1%).

Chitins are the second most abundant polysaccharide, usually found in fungi but not in mammals. Recently, studies performed on Chitins have demonstrated that it is dependent on complexity and size to initiate the recruitment and activation of cytokine production via MR, TLR-2 and Dectin 1. They are related to allergy-induced type 1 and 2 inflammation and have a great importance in pulmonary inflammation (4). A widely produced prostanoid within the body that plays an important role in the regulation of inflammatory responses – Prostaglandins E2 (PGE2) - is mainly involved in the inflammation signalling but also changes the activity of Antigen Presenting Cells (APCs) and T-cells, producing particular cytokines, thereafter influencing the functional properties of T-cell priming. T_h2 response inhibits the production of T_h2 by up-regulating IFN-ϒ and other cytokines. T_h17 is important in the protection against extracellular pathogens, i.e., fungi and bacteria. IL-1B signalling is pivotal in the early maturation of these T_h17 cells and studies have demonstrated that *C.albicans* induces a strong reaction. Another interesting aspect lies in the synergy between mannan and β-glucan. Mannans instigate PGE2 productions as the β-glucan works together via TLR2 (12). Observing graph C (fig 5), the analysis on the

data shows the significant difference between *A.niger* and the control (no cells) but not with the β-glucan, emphasising the similarity in responses of IL-1a produced when MARCO-/- MPI cells were stimulated by *C.albicans.* The possible explanation of this would be due to the similar make-up of the fungi and β-glucan. The β-glucan provided (Leiber), used similar make-up of the component to the one present in the fungus. Additionally, as mentioned before, the heat treatment disrupts and exposes more of the components of the cell wall of *C.albicans* and therefore would enhance the inflammatory response if the MARCO receptor as present (refer to figure 2 – graph D and F).

The results that were observed in figures 1, 2 and 3, β-glucan stimulated cells showed no significance compared to the control in little/no immune response. The reason why it is so is not known but a suggested solution is to prime the MPI cells with LPS initially and then expose them to β-glucan. As a result, it may lead to an enhanced response, unlike the experiment that has been done on this occasion.

As per the aim of this experiment, the importance of the MARCO receptor in the initiation of the innate immune response was tested in figures 4-8. The introduction mentioned the SR, known to fulfil a critical role in the host defence against bacteria, expresses high sensitivity MPI cells and AMs to *M. tuberculosis* according to the data published by Dr. Fejer, 2013 (5). AMs make up 95% of bronchial cells. They are active producers of cytokines and leukotriene's that have both pro- and anti-inflammatory roles, although they lack the ability to present the antigen on the cells (Antigen presenting cell-APC), down regulating the response of T-cells to pathogen. Nevertheless, macrophages are very efficient in recognising molecular patterns which occur in altered states or on pathogens (29). One specific feature of these receptors is the recognition of invariant structures on the various pathogens which is essential for the pathogenicity, differing from "self" antigens to avoid damage to the host- these pattern can either be found in body fluids, present as proteins and also as parts of the cell membrane. PRRs recognised by the macrophage include LPS, complement CTL- mannose receptor (MR) and class A SRs. Initially, MARCO SRs were known for the anti-bacterial response and not anti-fungal defence but studies done by Bin *et al* in 2003, demonstrated that there is direct recognition of major constituents of fungal cell walls (30, 31).

Figures 4-8, take into consideration all the different comparisons, and all of those, even visually, had no significant difference to deduce that an immune response was being induced. IL-6 levels, compared within the different species and also to the control showed no evidence that the cytokine was being released. This pattern followed for the IL-1β and also IL-1α (fig. 4- Graph B and C respectively). Statistical analysis was performed on each, but a highly interesting pattern of difference was noticed in Graph C (figure 4). Il-1α levels induced by *C.albicans* were significantly different to the levels produced by *A.niger* and the control. This difference was not seen between the levels of IL-1α produced in *C.albicans* and B-glucan stimulated MARCO-/- MPI cells. This trend became more obvious when the experiment was conducted on MARCO-/- cells than normal MPI cells, as there was nothing to induce an immune response. As mentioned before, this trend might be because both *C.albicans* and β-glucan have properties they share with the components of the cell wall, therefore, it can be suggested that they share some levels of expression when stimulated compared to other strains.

The MARCO-/- MPI cells that were stimulated were compared to the normal MPI cells that were stimulated earlier in the experiment. IL-6 and IL-1α were seen to show

differences in the amount of cytokines produced but IL-1β was not comparable when compared to all three stimuli (Figure 5-8). This demonstrates clearly that an immune response requires the MARCO receptor, but there may be other receptors e.g. TLRs and C-type lectins and Dectin-1 that detect components of fungi better than the MARCO SRs. Therefore, future experiments could look at the effect of other receptors that could affect the release of pro-inflammatory cytokines.

Limitations

There are various factors to consider when describing the limitations of the study. One of the limitations within the experiments conducted were the ELISA's as the concentrations of the reagents used could vary from one experiment to the other. This would lead to insufficient or overwhelming amounts of the reagents within the wells of the plate. Due to the constant need to dilute the primary and secondary antibodies, standard stocks for the different cytokines and Avidin, it required the calculations to be done which, even though checked by the supervisor, could lead to errors when considering the decimal point at which the figure was rounded up or down. Pipetting error can also be limiting factor, as only the materials provided were used. To avoid variation in pipetting, it can be ensured that the pipettes are all calibrate on a much more frequent basis.

In conclusion, the study showed clear evidence of the importance of the MARCO Scavenger Receptor in the initiation of an immune response to heat killed fungi. MPI cells that were used are a useful tool in the study of the how AMs recognise and build up a response towards invading infectious particles. There are many aspects that can be considered for the future experiments, for example, the other PRRs that recognise PAMPs within the lungs and how they play a protective or inhibition role in the pathogenicity of respiratory disorders. Studies done in the past have recognised that other receptors, including the mannose receptor (MR), complement 3 etc., bind to the fungal cells components of its capsule or cell wall.

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